#4

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

22469

Art Unit	: 1642	
Examiner	: G. Bansal	
Serial No.	: 09/939,483	
Filed	: August 24, 2001	
Inventors	: Fabrice DuPrat	
	: Florian LeSage	
	: Michel Fink	
	: Michel Lazdunski	Docket: 1201-CIP-DIV-2-00
Title	: FAMILY OF MAMMALIAN	Confirmation No.:
	: POTASSIUM CHANNELS,	
	: THEIR CLONING AND THEIR	
	: USE, ESPECIALLY FOR THE	
	: SCREENING OF DRUGS	Dated: September 13, 2001

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, DC 20231

Sir:

Prior to action on the merits, using clean copies of the Specification and Claims and also marked-up versions of such, we respectfully request consideration of the following amendments and remarks:

In the Specification (Marked-up Version)

Please replace the paragraph spanning pages 3 and 4 with the following:

The research activities that led to the cloning of the TWIK-1 and TASK channels was carried out in the manner described below with reference to the attached sequences and figures in which:

- SEQ ID [NO: 1] NO:1 represents the nucleotide sequence of the cDNA of TWIK1 and its deduced amino acid sequence.

- SEQ ID [NO: 2] NO:2 represents the amino acid sequence of the TWIK-1 protein.

- SEQ ID [NO: 3] NO:3 represents the nucleotide sequence of the cDNA of TASK and its deduced amino acid sequence.

- SEQ ID [NO: 4] NO:4 represents the amino acid sequence of the TASK protein.

On page 4, please replace the second paragraph with the following:

- Figure 1 represents the Northern blot analysis, the nucleotide sequences and the deduced amino acid sequence, as well as the hydrophobicity profile of TWIK-1 (SEQ ID NO:1). (a): expression of TWIK-1 mRNA in human tissues; each track contains 5 μg of poly(A)+RNA; the autoradiograph was exposed for 24 hours. (b) cDNA sequence of TWIK-1 and the amino acid sequences of the coding sequence. The supposed transmembranal segments are circled and the P domains are underlined: ❒ represents a potential glycosylation side and ■ represents the threonine residue in the consensus recognition site of protein kinaseC. (c): the hydrophobicity analysis and the topology of TWIK-1 deduced from it; the hydrophobicity values were calculated according to the method of Kyte and Doolittle (window size of 11 amino acids) and are presented in relation to the position of the amino acid; the shaded hydrophobic peaks correspond to the trans-membranal segments.

Please replace the paragraph spanning pages 4 and 5 with the following:

- Figure 2 represents the sequence alignments. (a): alignment of the P domains of TWIK-1, TOC/YORK and other representative K+ channel families (SEQ ID NO:9 through SEQ ID NO:23); the identical and conserved residues are circled in black and in gray, respectively. (b): alignment of TWIK-1 (SEQ ID NO:2) with potential homologs of; the sequences M110.2 (SEQ ID NO:7) and F17C8.5 (SEQ ID NO:6) were deduced from the gene sequences (respective access numbers Z49968 and Z35719); the

computerized splicing of the other genomic sequences of *C. elegans* (respective access numbers Z49889, P34411 and Z22180) is not sufficiently precise to allow their perfect alignment and is therefore not shown.

On page 7, please replace the first and second paragraphs with the following:

- Figure 8 (which consists of 8A and 8B, an enlargement of 8A) show the nucleotide and deduced amino acid sequences of human TASK (SEQ ID NO:3) and partial amino acid sequence of mouse TASK (SEQ ID NO:5). Consensus sites for N-linked glycosylation (*) and phosphorylation by protein kinase C (n), protein kinase A (s) and tyrosine kinase (1) in human TASK. These sites have been identified by using the prosite server (European Bioinformatics Institute) with the ppsearch software (EMBL Data library) based on the MacPattern program. The sequence of human and mouse TASK have been deposited in the GenBank/EMBL database under the accession numbers AF006823 and AF006824, respectively.

- Figure 9 shows the sequence comparison and membrane topology of TWIK-related channels. A: Alignment of human TWIK-1 (SEQ ID NO:2), mouse TREK-1 (SEQ ID NO:8) and human TASK (SEQ ID NO:4) sequences. Identical and conserved residues are shown in black and grey, respectively. Dashes indicate gaps introduced for a better alignment. Relative positions of putative transmembrane segments (M1 to M4) and P domains (P1 and P2) of human TASK are also indicated. The M1-M4 domains were deduced from a hydropathy profile computed with a window size of 11 amino acids according to the Kyte and Doolittle method (Kyte and Doolittle, 1982). B: Putative membrane topology of TWIK-1, TREK-1 and TASH channels.

Please replace the paragraph spanning pages 11 and 12 with the following:

The following characteristics of this K^+ channel were demonstrated:

- The sequences of the cDNA clones contain an ORE of 1011 nucleotides (SEQ ID NO:1) coding for a polypeptide of 336 amino acids (SEQ ID NO:2) shown in Figure 1b (SEQ ID NO:1).

- The protein has two P domains.

- Other than the P domains, no significant alignment was seen between TWIK-1 and a K^+ channel recently cloned in yeast and which also has two P domains (Ketchum, K.A. et al., 1995, Nature, 376, 690-695).

- Analysis of the hydrophobicity of TWIK-1, shown in Figure 1c, reveals the presence of four transmembranal domains, designated T1 to T4.

- By placing the NH_2 end on the cytoplasmic surface, in accordance with the absence of signal peptide, one obtains the topology model shown in Figure 1c.

- In this model, the two P domains are inserted in the membrane from the exterior in accordance with the known orientation of these loops in the K^+ channels.

- In addition, the general structural unit of TWIK-1 is similar to the unit that one would obtain by making a tandem of two classical subunits rectifying the entry of a potassium channel. Like a classical inward rectifier, TWIK-1 does not exhibit the highly conserved segment S4 which is responsible for the sensitivity to the membrane potential of the inward rectification of the K^+ channels of the Kv family.

- An unusual large loop of 59 amino acids is present between M1 and P1, such as to extend the length of the linker M1-P1 of the extrucellular side of the membrane.

- A potential site of N-glycosylation is present in this loop.

- Three consensus sites of phosphorylation are present at the N-terminal (Ser 19 for calcium calmodulin kinase II) and C-terminal (Ser 303 for casein kinase II) ends of the cytoplasmic domains, and in the M2-M3 linker (Thrl61 for protein kinase II).

- The alignment of the P domains of an important group of K^+ channels is presented in Figure 2a. It shows that the regions constituting the pore selective for K^+ are well conserved including the G residues in position 16 and 18 and three other residues indicating practically exclusively conservative changes in positions 7, 14 and 17. It is of interest to note that a leucine residue is present in the place of a tyrosine conserved in position 18 in the P2 domain of TWIK-1, or of a phenylalanine in position 17 of the P domain of the K^+ channel of type eag.

Please replace the paragraph spanning pages 14 and 15 with the following:

Single channel current recordings, shown in Figure 5, in an inside-out patch configuration or in a whole cell configuration show that the TWIK-1 channels assure the passage of influx or exit currents as a function, respectively, of a depolarization or a hyperpolarization (Figure 5a). The current voltage relationship of the single channel, shown in Figure 5b, shows a barely accentuated inward rectification in the presence of 3 mM (Figure 5) and 10 mM (not shown) of Mg^{2+} on the cytoplasmic side. As shown in Figure 5b, this rectification disappears in the absence of internal M^{2+}. With 3 mM of internal Mg^{2+}, the mean duration of opening at +80 mV is 1.9 ms and the unitary conductance is 19 +/- 1pS (Figure 5c). At-80 mV, the [charnels] channels are oscillating with a mean duration of opening of 0.3 ms, and a conductance value in creasing to 34 pS. The withdrawal of the internal Mg^{2+} ions does not influence the kinetic parameters under either polarized or depolarized conditions, but the unitary conductance measured at -80 mV

reaches 35 +/- 4 pS. This apparent increase in conductance in the single channel suggests that it is the extremely rapid oscillation induced by Mg^{2+} that results in an underestimation of the real value of conductance. The same properties were observed in the fixed cell configuration, showing that the channel behavior is not modified by the excision of the patch. The TWIK-1 channels in the excised patches do not discharge and do not appear to be deficient in intracellular constituents. In contrast to numerous channels which require the presence of ATP for their activity in the excised patch configuration, ATP is not required for the expression of TWIK-1. In addition, perfusion of the patch with a solution containing 10 mM of ATP does not induce any effect on the activity of the TWIK-1 channel.

Please replace the paragraph spanning pages 16 and 17 with the following:

TWIK-1 and TREK-1 sequences were used to search related sequences in GenBank database by using the Blast alignment program. There were identified two mouse Expressed Sequence Tag (EST, accession numbers W36852 and W36914) that overlap and give a contig fragment of 560 bp whose deduced amino acid sequence presents significant similarity with TWIK-1 and TREK-1. A corresponding DNA fragment was amplified by RT-PCR and used to screen a mouse brain cDNA library. Eight independent clones were isolated. The 1.8 kb cDNA insert of the longer one bears in its 5' part an open reading frame (ORF) coding for a 405 amino acids polypeptide (SEQ ID NO:5) (Figure 8). This ORF does not begin with an initiating methionine codon suggesting that the brain cDNA clones were partial. Ten additional positive clones were isolated from a mouse heart cDNA library. Analysis of their 5' sequence showed that all these clones were not longer than the clones previously isolated from brain. The 5' sequence has a very high GC

content and is probably associated to secondary structures that could have promoted prematurate stops of RNA reverse transcription during the construction of both mouse cDNA libraries. To overcome this problem, the complete cDNA was cloned in another species. The DNA probe was used to screen a cDNA library from human kidney, a tissue that express both TWIK-1 and TREK-1 channels. Two hybridizing clones were characterized. Both contain an ORF of 1185 nucleotides encoding a 394 amino acids polypeptide (SEQ ID NO:3 and SEQ ID NO:4) (Figure 8). The human protein sequence contains consensus sites for N-linked glycosylation (residue 53), and phosphorylation by protein kinase C (residues 358 and 383), tyrosine kinase (residue 323) and protein kinase A (residues 392 and 393). All these phosphorylation sites are located in the C-terminus part of the protein. Except for a 19 residues cluster (aa 276 to 294 in the human sequence), mouse and human proteins share a high overall sequence conservation (85% of identity) indicating that they probably are products of ortholog genes (Figure 8). Sequence alignments presented in Figure 9 clearly show that the cloned protein is a new member of the TWIK related K^+ channel family. Like TWIK-1 (SEQ ID NO:2) and TREK-1 (SEQ ID NO:8), TASK (SEQ ID NO:4) has four putative transmembrane segments (M1 to M4) and two P domains (P1 and P2) (Figures 9A and 9B). TASK is 58 amino acids longer than TWIK-1 and 24 amino acids longer than TREK-1 because its C-terminus is more extended.

Please replace the paragraph spanning pages 18 and 19 with the following:

The TASK distribution was further studied in adult mouse brain and heart by *in situ* hybridization. *In situ* hybridization experiments were performed on adult Balb/c mice by using standard procedures (Fink et al., 1996b). An antisense oligonucleotide (48 mer, 5'-

CACCAGCAGGTAGGTGAAGGTGCACACGATGAGAGCCAACGTGCGCAC-3') (SEQ ID NO:24) complementary to the mouse cDNA sequence of TASK (from nucleotides 7 to 54) was used to detect the expression of TASK transcripts in frozen fixed brain sections (10 μm). The probe was 3'-end-labelled with (α-^{33}P)dATP. Sections were digested with 5 μg/ml of proteinase K for 15 min at 37°C, acetyled for 10 min in 0.25% acetic anhydre in 0.1 M triethanolamine. Hybridization was carried out overnight at 37°C in 2X SSC, 50% formamide, 10% dextran sulfate, 1X Denhardt's solution, 5% sarcosyl, 500 μg denatured salmon sperm DNA, 250 mg/ml yeast tRNA, 20 mM dithiothreitol, and 20 mM $NaPO_4$ with 0.2 ng/ml of radiolabelled probe (specific activity = 8.10^8 dpm/μg). Slides were then washed in 1X SSC before dehydratation, drying, and apposition to hyperfilm-ßmax (Amersham) for 6 days. The specificity of labelling was verified by *in situ* hybridization using cold displacement of radioactive probe with a 500-fold excess of unlabelled oligonucleotide.

Please replace the first full paragraph on page 23 with the following:

Comparison of the complete sequence of TWIK-1 with the sequences of the Genbank data base allowed identification of at least five genes of *Caenorhabditis elegans*, which had been characterized in the context of the Nematode Sequencing project, which may encode additional structural homologues of TWIK-1. The alignment of two of these homologues (SEQ ID NO:7 and SEQ ID NO:6) with TWIK-1 (SEQ ID NO:2) is shown in Figure 2b. The degree of similarity between the deduced protein sequences of *C. elegans* and TWIK-1 are approximately 55 to 60%. Amino acid sequence identities among the deduced polypeptide sequences range from 25 to 28%. Interestingly, the degree of si9milarity and amino acid sequence identity of the various *C. elegans* are not greater than

what was determined for TWIK-1. These results indicate that other TWIK-1 relates potassium channels may be present in the *C. elegans* genome and suggest that additional members of the TWIK-1 [famiy] family of potassium channels may exist in mammals.

Please replace the paragraph spanning pages 23 and 24 with the following:

This invention describes the isolation and the characterization a novel human K^+ channel. This channel has an overall [structure] structural similarity with TWIK-1 and TREK-1 channels that suggests a common ancestral origin. Despite this similar structural organization, the amino acid identity between TASK and the two other mammalian related channels is very low (25-28%). Sequence homologies are no higher between TASK and a recently cloned *Drosophila* channel that also belongs to the structural TWIK channel class (Goldstein et al., 1996). The highest degree of sequence conservation is in the two P domains and the M2 segment. In these regions the amino acid identity reaches ~50%. Like other TWIK-related channels, TASK contains an extended M1P1 interdomain. This peculiar domain has been shown to be extracellular in the case of TWIK-1 and to be important for the self-association of two TWIK-1 subunits. The TWIK-1 homodimers are covalent because of the presence of an interchain disulfide bridge between cysteines 69 located in the M1P1 interdomain (Lesage et al., 1996b). This particular cysteine residue is conserved in TREK-1 (residue 93) (SEQ ID NO:8) but not in TASK strongly suggesting that TASK probably does not form covalent dimers as observed for TWIK-1 (Lesage et al., 1996b) and TREK-1 (unpublished data).

Please replace the paragraph spanning pages 28 and 29 with the following:

The present invention also has as its object a new family of K^+ channels, of which TWIK-1 and TASK are members, which polypeptides are present in most human tissues,

but especially abundant in the brain and the heart, and which exhibit the properties and structure of the type of those of the TWIK-1 channels described above. Thus, the invention relates to an isolated, purified protein whose amino acid sequence is represented in the attached sequence list as [number] SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:[X]5, or a functionally equivalent derivative of these sequences. Such derivatives can be obtained by modifying and/or suppressing one or more amino acid residues of this sequence or by segmenting this sequence, as long as this modification and/or suppression or deletion of a fragment does not modify the functional properties of the TASK type potassium channel of the resultant protein.

Please delete first page 30, lines 5 - 20.

Please replace the first full paragraph on second page 30 with the following:

For Northern blot analysis, poly(A)$^+$ RNAs were isolated from adult mouse tissues and blotted onto nylon membranes as previously described (Lesage et al., 1992). The blot was probed with the ^{32}P-labelled insert of pBS-mTASK in 50% Formamide, 5X SSPE (0.9 M sodium chloride, 50 mM sodium phosphate (pH 7.4), 5 mM EDTA), 0.1% SDS, 5X Denhardt's solution, 20 mM potassium phosphate (pH 6.5) and 250 μg denatured salmon sperm DNA at 50°C for 18h and washed stepwise at 55°C to a final stringency of 0.2X SSC, 0.3% SDS. For hybridization of human multiple tissues Northern blots from Clontech, the procedure was identical except that the probe was derived from pBS-hTASK. The cDNA insert of pBS-hTASK contains different repeat sequences (AluJb, MIR and (CGG)n) in the untranslated regions (UTR) and a SmaI/ApaI restriction fragment of 1390 bp spanning the coding sequence was chosen as a [p30robe] probe that does not contain these repeats.

In the Claims (Marked-up Version)

31. (Amended) The transgenic animal of claim 29 which overexpresses the potassium transport channel encoded by the nucleic acid sequence represented by SEQ ID [No. 3] NO:3.

32. (Amended) The transgenic animal of claim 29 which is deficient in the expression of the potassium transport channel encoded by the nucleic acid sequence represented by SEQ ID [No. 3] NO: 3.

Remarks

We have amended the Specification and the Claims to correct minor typographical errors and to place them into form for examination on the merits.

Passage to the appropriate art unit for examination on the merits is respectfully requested.

Respectfully submitted,

T. Daniel Christenbury
Reg. No. 31,750

TDC:lh
(215) 563-1810

In the Specification (Clean copy as amended)

Please replace the paragraph spanning pages 3 and 4 with the following:

The research activities that led to the cloning of the TWIK-1 and TASK channels was carried out in the manner described below with reference to the attached sequences and figures in which:

- SEQ ID NO:1 represents the nucleotide sequence of the cDNA of TWIK1 and its deduced amino acid sequence.

- SEQ ID NO:2 represents the amino acid sequence of the TWIK-1 protein.

- SEQ ID NO:3 represents the nucleotide sequence of the cDNA of TASK and its deduced amino acid sequence.

- SEQ ID NO:4 represents the amino acid sequence of the TASK protein.

On page 4, please replace the second paragraph with the following:

- Figure 1 represents the Northern blot analysis, the nucleotide sequences and the deduced amino acid sequence, as well as the hydrophobicity profile of TWIK-1 (SEQ ID NO:1). (a): expression of TWIK-1 mRNA in human tissues; each track contains 5 μg of poly(A)+RNA; the autoradiograph was exposed for 24 hours. (b) cDNA sequence of TWIK-1 and the amino acid sequences of the coding sequence. The supposed transmembranal segments are circled and the P domains are underlined: ❒ represents a potential glycosylation side and ■ represents the threonine residue in the consensus recognition site of protein kinaseC. (c): the hydrophobicity analysis and the topology of TWIK-1 deduced from it; the hydrophobicity values were calculated according to the method of Kyte and Doolittle (window size of 11 amino acids) and are presented in relation to the position of the amino acid; the shaded hydrophobic peaks correspond to the trans-

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- Analysis of the hydrophobicity of TWIK-1, shown in Figure 1c, reveals the presence of four transmembranal domains, designated T1 to T4.

- By placing the NH_2 end on the cytoplasmic surface, in accordance with the absence of signal peptide, one obtains the topology model shown in Figure 1c.

- In this model, the two P domains are inserted in the membrane from the exterior in accordance with the known orientation of these loops in the K^+ channels.

- In addition, the general structural unit of TWIK-1 is similar to the unit that one

would obtain by making a tandem of two classical subunits rectifying the entry of a potassium channel. Like a classical inward rectifier, TWIK-1 does not exhibit the highly conserved segment S4 which is responsible for the sensitivity to the membrane potential of the inward rectification of the K^+ channels of the Kv family.

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- Three consensus sites of phosphorylation are present at the N-terminal (Ser 19 for calcium calmodulin kinase II) and C-terminal (Ser 303 for casein kinase II) ends of the cytoplasmic domains, and in the M2-M3 linker (Thrl61 for protein kinase II).

- The alignment of the P domains of an important group of K^+ channels is presented in Figure 2a. It shows that the regions constituting the pore selective for K^+ are well conserved including the G residues in position 16 and 18 and three other residues indicating practically exclusively conservative changes in positions 7, 14 and 17. It is of interest to note that a leucine residue is present in the place of a tyrosine conserved in position 18 in the P2 domain of TWIK-1, or of a phenylalanine in position 17 of the P domain of the K^+ channel of type eag.

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mM (Figure 5) and 10 mM (not shown) of Mg^{2+} on the cytoplasmic side. As shown in Figure 5b, this rectification disappears in the absence of internal M^{2+}. With 3 mM of internal Mg^{2+}, the mean duration of opening at +80 mV is 1.9 ms and the unitary conductance is 19 +/- 1pS (Figure 5c). At-80 mV, the channels are oscillating with a mean duration of opening of 0.3 ms, and a conductance value in creasing to 34 pS. The withdrawal of the internal Mg^{2+} ions does not influence the kinetic parameters under either polarized or depolarized conditions, but the unitary conductance measured at -80 mV reaches 35 +/- 4 pS. This apparent increase in conductance in the single channel suggests that it is the extremely rapid oscillation induced by Mg^{2+} that results in an underestimation of the real value of conductance. The same properties were observed in the fixed cell configuration, showing that the channel behavior is not modified by the excision of the patch. The TWIK-1 channels in the excised patches do not discharge and do not appear to be deficient in intracellular constituents. In contrast to numerous channels which require the presence of ATP for their activity in the excised patch configuration, ATP is not required for the expression of TWIK-1. In addition, perfusion of the patch with a solution containing 10 mM of ATP does not induce any effect on the activity of the TWIK-1 channel.

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located in the M1P1 interdomain (Lesage et al., 1996b). This particular cysteine residue is conserved in TREK-1 (residue 93) (SEQ ID NO:8) but not in TASK strongly suggesting that TASK probably does not form covalent dimers as observed for TWIK-1 (Lesage et al., 1996b) and TREK-1 (unpublished data).

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The present invention also has as its object a new family of K^+ channels, of which TWIK-1 and TASK are members, which polypeptides are present in most human tissues, but especially abundant in the brain and the heart, and which exhibit the properties and structure of the type of those of the TWIK-1 channels described above. Thus, the invention relates to an isolated, purified protein whose amino acid sequence is represented in the attached sequence list as SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:5, or a functionally equivalent derivative of these sequences. Such derivatives can be obtained by modifying and/or suppressing one or more amino acid residues of this sequence or by segmenting this sequence, as long as this modification and/or suppression or deletion of a fragment does not modify the functional properties of the TASK type potassium channel of the resultant protein.

Please delete first page 30, lines 5 - 20.

Please replace the first full paragraph on second page 30 with the following:

For Northern blot analysis, poly(A)$^+$ RNAs were isolated from adult mouse tissues and blotted onto nylon membranes as previously described (Lesage et al., 1992). The blot was probed with the ^{32}P-labelled insert of pBS-mTASK in 50% Formamide, 5X SSPE (0.9 M sodium chloride, 50 mM sodium phosphate (pH 7.4), 5 mM EDTA), 0.1% SDS, 5X Denhardt's solution, 20 mM potassium phosphate (pH 6.5) and 250 μg denatured salmon

sperm DNA at 50°C for 18h and washed stepwise at 55°C to a final stringency of 0.2X SSC, 0.3% SDS. For hybridization of human multiple tissues Northern blots from Clontech, the procedure was identical except that the probe was derived from pBS-hTASK. The cDNA insert of pBS-hTASK contains different repeat sequences (AluJb, MIR and (CGG)n) in the untranslated regions (UTR) and a SmaI/ApaI restriction fragment of 1390 bp spanning the coding sequence was chosen as a probe that does not contain these repeats.

In the Claims (Clean copy as amended)

31. (Amended) The transgenic animal of claim 29 which overexpresses the potassium transport channel encoded by the nucleic acid sequence represented by SEQ ID NO:3.

32. (Amended) The transgenic animal of claim 29 which is deficient in the expression of the potassium transport channel encoded by the nucleic acid sequence represented by SEQ ID NO: 3.